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Interleukin (IL)-5 Downregulates Tumor Necrosis Factor (TNF)-Induced Eotaxin Messenger RNA (mRNA) Expression in Eosinophils

Induction of Eotaxin mRNA by TNF and IL-5 in Eosinophils

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An eotaxin is a chemoattractant specific for eosinophils that are known to play a role in helminth infection and allergic responses. Although several cellular sources have been reported to produce eotaxin, it would be interesting to know whether eosinophils are able to produce their own eotaxin and participate in recruitment of themselves in response to inflammation. To this end, a cloned eotaxin complementary DNA was transcribed *in vitro* to use as a probe for detecting eotaxin messenger RNA (mRNA), and eotaxin protein levels were determined by enzyme-linked immunosorbent assay. Eotaxin mRNA was, as analyzed by *in situ* hybridization, rarely detectable in unstimulated eosinophils, but was strongly induced in eosinophils when stimulated with tumor necrosis factor (TNF). Interleukin (IL)-5, which is known to be a major factor of eosinophil survival *in vivo* and *in vitro*, was also able to induce a modest level of eotaxin mRNA but inhibited TNF-induced eotaxin mRNA expression in a dose-response manner. Dexamethasone inhibited TNF-induced eotaxin mRNA expression. This result was consistent with that from reverse transcription/polymerase chain reaction followed by Southern blot analysis. Unlike the little expression of eotaxin mRNA in the absence of stimuli, the measurement of eotaxin protein revealed that a considerable amount of eotaxin protein was constitutively produced in unstimulated eosinophils. Its expression was upregulated by TNF and IL-5 as well. However, the inhibitory effect of IL-5 on TNF-mediated eotaxin protein production was not as pronounced as that on eotaxin mRNA induction. Collectively, these data reflect the complex physiology of eosinophils in the expression of eotaxin gene upon the exposure to their survival and/or death factors. Han, S. J., J. H. Kim, Y. J. Noh, H. S. Chang, C. S. Kim, K.-S. Kim, S. Y. Ki, C. S. Park, and I. Y. Chung. 1999. Interleukin (IL)-5 downregulates tumor necrosis factor (TNF)-induced eotaxin messenger RNA (mRNA) expression in eosinophils: induction of eotaxin mRNA by TNF and IL-5 in eosinophils. *Am. J. Respir. Cell Mol. Biol.* 21:303-310.

Eosinophil is known to be an important effector cell in allergic inflammations and parasitic infections by releasing toxic granules into target tissues (1, 2). Several reports have demonstrated that eosinophils are accumulated in peripheral blood, bronchoalveolar lavage fluid of asth-

matic patients, and airway of allergen-sensitized animals (3-5). Eosinophil trafficking is regulated by a wide variety of chemotactic cytokines or chemotactic factors (4, 6-8). Eotaxin is a CC chemokine that selectively recruits eosinophils, unlike other chemoattractants of eosinophils, and binds CC chemokine receptor 3 in eosinophils (9-12), basophils (13), and the T-cell subpopulation (14). A variety of tissues and cell types have been shown to produce eotaxin. Eotaxin messenger RNA (mRNA) and/or protein are detected in several human tissues, such as small intestine and colon, at high levels (15, 16) as well as in epithelial cells, monocytes, lymphocytes, and eosinophils themselves present in bronchial epithelium and nasal polyps of patients with asthma (17).

Proinflammatory cytokines, including tumor necrosis factor (TNF), interleukin (IL)-1, and interferon (IFN)- γ , are released in the early stage of allergic inflammation. An important function of TNF is to induce an influx of eosino-

(Received in original form June 29, 1998 and in revised form February 8, 1999)

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Abbreviations: complementary DNA, cDNA; dexamethasone, Dex; digoxigenin, DIG; enzyme-linked immunosorbent assay, ELISA; granulocyte macrophage colony-stimulating factor, GM-CSF; interleukin, IL; messenger RNA, mRNA; phosphate-buffered saline, PBS; reverse transcription/polymerase chain reaction, RT-PCR; tumor necrosis factor, TNF; uridine triphosphate, UTP.

Am. J. Respir. Cell Mol. Biol. Vol. 21, pp. 303-310, 1999
Internet address: www.atsjournals.org

phils into tissues through the increased expression of adhesion molecules on endothelial and epithelial cells (18–22). TNF also induces the synthesis of granulocyte macrophage colony-stimulating factor (GM-CSF) and regulated on activation, normal T cells expressed and secreted (23–26). Among its many effects, GM-CSF can stimulate the production, maturation, and activation of eosinophils (27–29). Endogenously released TNF has been shown to contribute to allergen-induced airway eosinophilia and hyperresponsiveness (30), partly through induction of nitric oxide synthases in bronchial epithelium (31) and eosinophil degranulation (2, 32, 33). Although eotaxin mRNA and protein tend to be expressed constitutively in several cell types, its expression may also be regulated in response to proinflammatory cytokines in cell lines. IL-1, TNF, and IFN- γ induce eotaxin mRNA in lung epithelial cell lines A549 and BEAS 2B (34), cultured endothelial cells (35), the monocytic cell line (36), and dermal fibroblasts (37). IL-5 is known to be responsible not only for early and late stages of eosinophil differentiation but also for prolongation of eosinophil survival through IL-5 receptor activation followed by phosphorylation of SH2 protein tyrosine phosphatase 2 (38). IL-5 enhances the level of blood eosinophils in concert with eotaxin by facilitating the mobilization of eosinophils from the bone-marrow pool into blood, and thus can cause eosinophilia (8, 39). Further, IL-5 causes normodense eosinophils to become hypodense (40), which is believed to be activated, and directly activates eosinophil degranulation. Little is known, however, about whether TNF and/or IL-5 would affect expression of the eotaxin gene itself in eosinophils, although chemotactic factors (41) and IL-3 (42) are reported to upregulate or induce eosinophil-derived eotaxin.

In the present study, we examined the effect TNF and/or IL-5 on eotaxin mRNA and protein expression in peripheral blood eosinophils. Our results demonstrate that either TNF or IL-5 alone induces/augments eotaxin mRNA protein, and that IL-5 significantly inhibits TNF-induced eotaxin mRNA in a dose-dependent fashion. Eotaxin protein production was detected in unstimulated eosinophils and regulated by these cytokines, yet to a lesser extent than eotaxin mRNA expression. These results suggest that there is a complex communication between TNF and IL-5 in terms of eotaxin synthesis.

Materials and Methods

Reagents

TNF and IL-5 were purchased from R&D Systems (Minneapolis, MN) and their activities were described by the supplier. Dexamethasone (Dex) was from Sigma Chemical Co. (St. Louis, MO). NBT (4-nitro-tetrazolium-chloride) and BCIP (5-bromo-4-chloro-3-indoyl-phosphate) were obtained from Boehringer Mannheim (Mannheim, Germany). Human serum albumin, RPMI 1640, and *in vitro* transcription kits were from GIBCO BRL (Gaithersburg, MD). Restriction enzymes were obtained from New England Biolabs, Inc. (Beverly, MA). Percoll stock was purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). Diff-Quik solution was obtained from American Scientific Products (McGaw Park, IL).

Purification of Eosinophils

Eosinophils were enriched from blood of atopic patients by the discontinuous Percoll gradient method (43). Briefly, heparinized venous blood was sedimented with 5% dextran in 0.9% NaCl at room temperature for 60 min to remove erythrocytes. A leukocyte-rich layer was aspirated, centrifuged at $300 \times g$ for 10 min, and then washed twice in [1,4-piperazinebis (ethane sulfonic acid)] (Pipes) buffer (25 mM Pipes, 110 mM NaCl, 5 mM KCl, 40 mM NaOH, and 5.4 mM glucose). Percoll and Pipes buffer were mixed to obtain solutions of the following densities (in g/ml): 1.100 (1.5 ml), 1.090 (3 ml), 1.085 (3 ml), and 1.080 (3 ml). The osmolarity of Percoll ranged from 280 to 310 mosm/kg, pH 7.4. The solutions were then layered at the indicated volumes in 140×10 mm polystyrene tubes. Cells were suspended in 2 ml of the Percoll solution (1.070 g/ml) and were layered on top of the gradients and centrifuged at $1,600 \times g$ for 30 min at 4°C. The fractions containing eosinophils were pooled and washed twice in RPMI 1640 medium. The eosinophil-enriched fractions were incubated with CD16 monoclonal antibody-conjugated microbeads (Miltényi Biotec, Belgisch-Gladbach, Germany), and negative selection was carried out with a magnetic cell separator (MACS system; Becton-Dickinson, Mountain View, CA) to remove the contaminated neutrophils (44). The purity of eosinophils was more than 98% as determined on light microscopic examination of cytocentrifuge slides prepared by Diff-Quik stain. Viability of eosinophils was determined by exclusion of propidium iodide (2 g/ml) with a flow cytometer (FACScan; Becton-Dickson) (45). The viability was more than 98% at the beginning of culture.

RNA Isolation

Total cellular RNA was isolated from the purified eosinophils that had been treated with TNF or IL-5 or both for 4 h. RNA isolation followed the procedure of Chomczynski and Sacchi (46) using Tri-reagent (Molecular Research Center, Cincinnati, OH). Briefly, cells were washed twice and pelleted. RNA was extracted with guanidium isothiocyanate and phenol, and precipitated with ethanol.

Isolation and Cloning of a Human Eotaxin Gene

Human eotaxin complementary DNA (cDNA) was isolated from total RNA of eosinophils using a reverse transcription/polymerase chain reaction (RT-PCR). Briefly, 5 μ g of total RNA was reverse transcribed by 200 U of Moloney mouse leukemia virus reverse transcriptase (GIBCO BRL) at 42°C for 50 min. The resulting cDNA mixture was amplified with 2.5 U of Ampli Taq DNA polymerase (Boehringer Mannheim) in a final volume of 50 μ l containing 20 mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl₂, 1 mM of each deoxynucleotide triphosphate, 0.01 M dithiothreitol, and 50 pmol of each primer. Two oligonucleotide primers were synthesized on the basis of the published sequence of human eotaxin cDNA (15). The upstream primer (GGGCCAGCTTCTGTCCCAAC) is complementary to positions 168–187 of the 5' end of the human eotaxin cDNA, and the downstream primer (TTATGCTTTGGAGTTGGAGATTT) is complementary to positions 369–392 of the 3' end of the human eotaxin gene.

Amplification was performed in a thermocycler (Perkin-Elmer TC-1; Perkin-Elmer, Branchburg, NJ) for 40 cycles with the initial denaturation at 94°C for 5 min and the final extension steps at 72°C for 10 min (one cycle = 1 min at 94°C, 2 min at 57°C, and 2 min at 72°C). The PCR product (225 base pairs [bp]) was subcloned into pGEM-T vector (Promega, Madison, WI) and its sequence was confirmed by DNA sequencing.

In Vitro Transcription

The recombinant plasmid was linearized with *Bam*HI or *Nde*I for the production of sense and antisense RNA, respectively. *In vitro* transcription was performed using a digoxigenin (DIG)-11-uridine triphosphate (UTP) RNA labeling kit (Boehringer Mannheim). Briefly, 1 µg of plasmid was transcribed in a final volume of 20 µl containing 40 mM Tris-HCl (pH 7.5); 6 mM MgCl₂; 2 mM spermidine; 10 mM NaCl; 10 mM dichlorodiphenyltrichloroethane; 20 U ribonuclease inhibitor; one-tenth volume of 10× NTP labeling mixture containing 10 mM each of adenosine triphosphate, cytidine triphosphate, and guanosine triphosphate; 6.5 mM UTP; 3.5 mM of DIG-UTP; and 20 U of SP6 RNA polymerase for sense RNA or 20 U of T7 RNA polymerase for antisense RNA at 37°C for 2 h. The resulting DIG-labeled eotaxin transcript was then purified by electrophoresis in 5% polyacrylamide/8 M urea; excised from the gel after staining with ethidium bromide; eluted overnight at 37°C in 300 µl of elution buffer containing 0.5 M NH₄OAc, 1 mM ethylenediaminetetraacetic acid, and 0.1% sodium dodecyl sulfate; and titrated on nitrocellulose membrane (Amersham, Arlington Heights, IL) for the use of proper amount. The eotaxin antisense riboprobe for Southern blot analysis was also prepared using T7 RNA polymerase under the same conditions used for preparation of the DIG-labeled RNA by including 12.5 µM α-[³²P]UTP (800 Ci/mmol, 40 mCi/ml) (Arlington Heights, IL) instead of UTP and DIG-UTP, as previously described (47).

In Situ Hybridization

In situ hybridization was performed with a DIG-RNA labeling kit according to the manufacturer's instructions (Boehringer Mannheim). The cultured eosinophils were prepared on poly-L-lysine-treated slides and fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) (pH 7.4) for 5 min at 4°C, then rinsed twice in diethylpyrocarbonate-treated distilled water. The slides were dehydrated by sequential treatment with ethanol and then incubated at 42°C overnight in 20 µl of the hybridization mixture containing 2 µg/ml of DIG-labeled probe. After washing and blocking, the eosinophils were incubated with anti-DIG-alkaline phosphatase (AP) antibody (3 U/ml) (1:500 dilution) and visualized by reaction in NBT and BCIP solutions (48).

RT-PCR and Southern Blot

RT-PCR was carried out using the same protocol as described earlier except for cycle numbers. The amount of 1 µg of total RNA isolated from eosinophils that had been treated with TNF and/or IL-5 was used as a template to synthesize cDNA. The resulting cDNA mixture was amplified using the same primers described earlier. Amplifi-

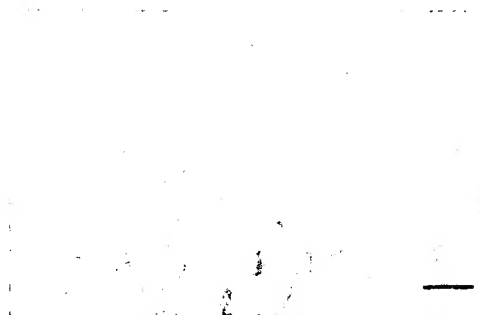
cation was performed in a thermocycler for 28 cycles with the initial denaturation at 94°C for 5 min and the final extension steps at 72°C for 10 min (one cycle = 1 min at 94°C, 2 min at 57°C, and 2 min at 72°C). The amount of 5 µl of the resulting PCR product (225 bp) was applied to a 1.5% agarose gel electrophoresis, and visualized by Southern blot hybridization with [³²P]-labeled eotaxin antisense riboprobe. As a control, a fragment of β-actin gene was amplified under the same conditions using a set of primers: upstream primer, TAAGCTTAAGGAGA-AGCTGTGCTACG; and downstream primer, GGGATC-CACGTCACACTTCAT.

Enzyme-Linked Immunosorbent Assay

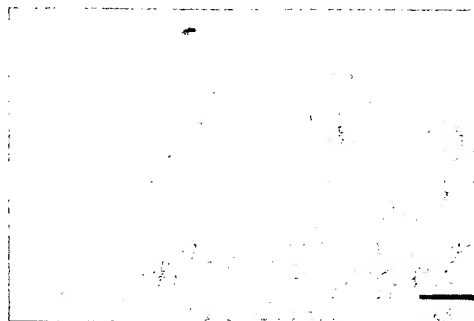
A sandwich enzyme-linked immunosorbent assay (ELISA) was employed for measurement of human eotaxin protein. Each well of a 96-well microplate (Nunc, Roskilde, Denmark) was coated with 3 µg/ml of an antihuman eotaxin monoclonal antibody (R&D Systems) and incubated at 4°C overnight. After the plates were incubated with a blocking solution (3% bovine serum albumin and 0.3% Tween 20 in PBS) at room temperature for 30 min and washed, the culture supernatants or cell lysates (100 µl) were added. After washing, 333 ng of a rabbit antihuman eotaxin polyclonal antibody (Pepro Tech, Inc., Rocky Hill, NJ) was added to each well, followed by incubation at room temperature for 2 h. A total of 100 µl of a horseradish peroxidase-conjugated goat antirabbit immunoglobulin antibody (Organon Teknica Corp., Durham, NC) (1:3,000 dilution) in PBS was added to each well and the plates were incubated at room temperature for 1 h. After washing, color was developed in the presence of 3,3',5,5'-tetramethylbenzidine substrate according to the instructions of the manufacturer (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD), and then optical density was measured at 450 nm. The standard curve was generated using recombinant human eotaxin (R&D Systems). The lower-limit eotaxin sensitivity under this condition was approximately 30 pg/ml human recombinant eotaxin.

Results

Eosinophils were isolated and stained with Diff-Quik solution. As in Figure 1g, both purity and viability were more than 98%, indicating a highly homogeneous population of eosinophils and their integrity. The cells were incubated with either TNF (10 ng/ml) or IL-5 (1 ng/ml) and were then subjected to *in situ* hybridization procedure. The TNF-treated eosinophils were not stained with the substrates of alkaline phosphatase when hybridized with sense riboprobe as a negative control (Figure 1a). Untreated eosinophils expressed little or no detectable eotaxin mRNA as analyzed with antisense riboprobe (Figure 1b), suggesting that unstimulated eosinophils do not constitutively produce eotaxin mRNA, or produce little, if any. When the cells were stimulated with TNF (10 ng/ml) for 4 h, the number of eosinophils expressing eotaxin mRNA was markedly increased (Figure 1c). In contrast, a modest increase in eosinophils expressing eotaxin mRNA was observed when eosinophils were stimulated with IL-5 (1.0 ng/ml) (Figure 1d). Comparable results were obtained in re-



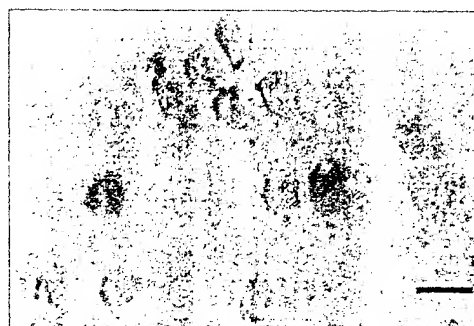
(a) Control (s)



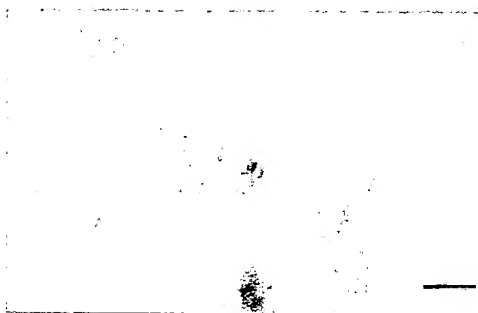
(b) Control



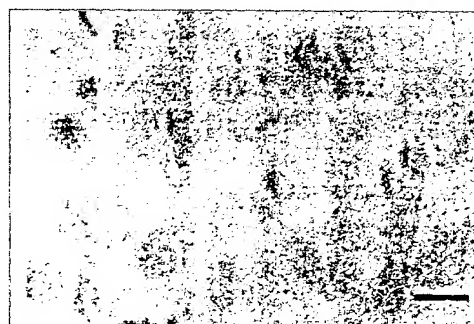
(c) TNF



(d) IL-5



(e) TNF + IL-5



(f) TNF + Dex



(g) Diff-Quik staining

Figure 1. In situ hybridization of eotaxin mRNA in eosinophils. Eosinophils were incubated for 4 h with medium (a and b; sense and antisense riboprobes, respectively), or TNF (10 ng/ml) (c), or IL-5 (1 ng/ml) (d), or TNF plus IL-5 (e), or TNF plus Dex (20 μ M) (f). Cells were isolated, fixed, and then hybridized with DIG-labeled eotaxin sense or antisense riboprobes. The presence of eotaxin mRNA was visualized by incubation of anti-DIG-AP antibody. Alternatively, eosinophils were directly stained with Diff-Quik (g). The scale bars indicate 10 μ m. These results represent one set of three separate experiments.

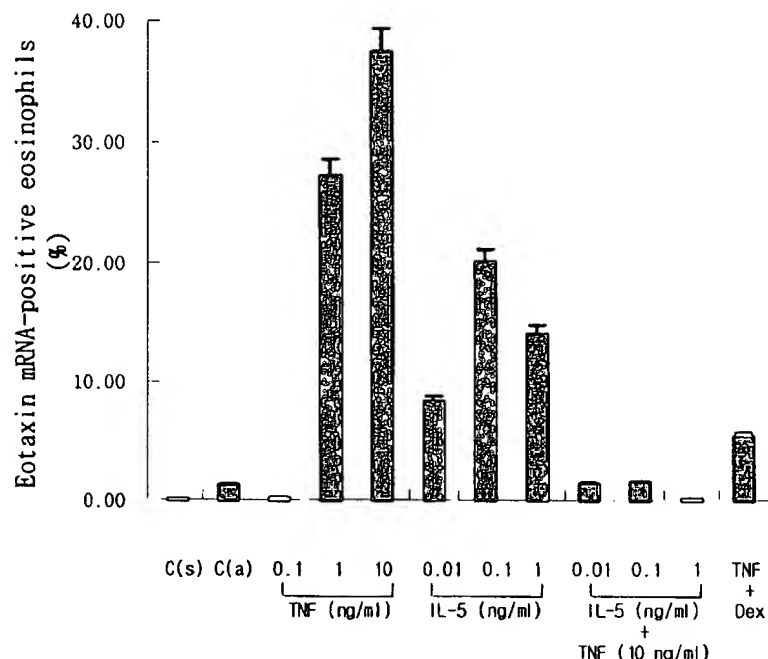


Figure 2. Effect of IL-5 on TNF-induced eotaxin mRNA in eosinophils. Eosinophils were incubated for 4 h with different concentrations of TNF (0.1 to 10 ng/ml) or IL-5 (0.01 to 1.0 ng/ml), or IL-5 (0.01 to 1.0 ng/ml) in the presence of TNF (10 ng/ml), or TNF plus Dex (20 μ m). The cells were then subjected to *in situ* hybridization as described in MATERIALS AND METHODS. The percentage of eotaxin-positive eosinophils was determined microscopically by the counting of stained cells in five fields in a blind manner by one person. The data presented here are representative of three separate experiments.

sponse to different concentrations of IL-5 from 0.01 ng/ml to 1.0 ng/ml (data not shown). When eosinophils were treated with both TNF (10 ng/ml) and IL-5 (1 ng/ml) simultaneously, eotaxin mRNA-positive cells were strikingly reduced (Figure 1e), compared with those induced by either TNF or IL-5 alone. This effect was also observed in Dex-treated eosinophils, as expected (Figure 1f). To further verify the inhibition of TNF-induced eotaxin mRNA expression by IL-5, eosinophils were stimulated with different concentrations of IL-5 in the presence of TNF. As shown in Figure 2, eotaxin mRNA-expressing eosinophils were 37.4 ± 2.1 , 27.2 ± 2.5 , and $0.3 \pm 0.1\%$ upon exposure to TNF alone in the concentrations of 10, 1.0, and 0.1 ng/ml, respectively; whereas those cells were 14.0 ± 1.4 , 20.1 ± 2.6 , and $8.4 \pm 0.5\%$ in response to IL-5 alone in the concentrations of 1.0, 0.1, and 0.01 ng/ml, respectively. In the presence of both TNF (10 ng/ml) and IL-5, however, the numbers of eotaxin mRNA-expressing eosinophils were considerably decreased. Thus, TNF-induced eotaxin mRNA expression was significantly inhibited by IL-5 in a dose-dependent manner. A similar result was obtained from RT-PCR followed by Southern blot hybridization (Figure 3) in which PCR was performed in 28 cycles to stay in the exponential phase of amplification. To measure eotaxin protein levels, we performed ELISA using two different antieotaxin antibodies. Under these conditions the detectable range of eotaxin was 30 to 3,000 pg/ml. The eotaxin protein was not detectable in culture supernatants from eosinophil. Consistent with this result, we were also unable to detect eotaxin protein by Western blot using the antieotaxin polyclonal antibody used in ELISA. As shown in Figure 4, a substantial amount of eotaxin protein was detected in cell lysate prepared from unstimulated eosinophils (approximately 100 pg/ 10^7 cells), increased up to 8 h, and decreased thereafter, probably

due to cell death in *in vitro* culture. The protein levels (a few hundred pg/ 10^7 cells at maximum) parallel those seen in a recent study, in which freshly isolated eosinophils released a few dozen picograms per 10^6 cells (41). Either

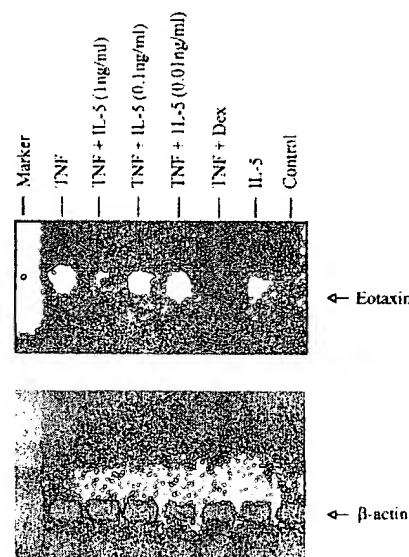
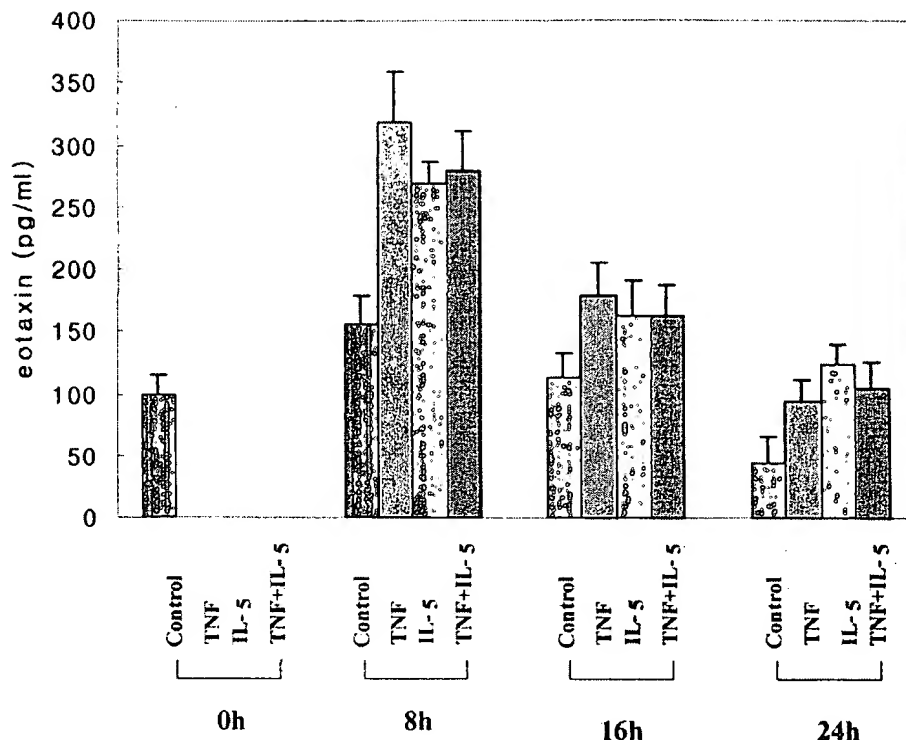


Figure 3. Demonstration of eotaxin mRNA in stimulated eosinophils by RT-PCR. Eosinophils were incubated for 4 h with TNF (10 ng/ml), or with different concentrations of IL-5 (0.01, 0.1, and 1.0 ng/ml) in the presence of TNF (10 ng/ml), or with TNF (10 ng/ml) plus Dex (20 μ m). Total cellular RNA (1- μ g aliquots) was isolated and then processed in RT-PCR as described in MATERIALS AND METHODS. The 5- μ l aliquots of each resulting PCR product were analyzed with a [32 P]UTP-labeled human eotaxin antisense riboprobe. The blot was exposed on X-Omat film for 8 h at -70°C .

Figure 4. Eotaxin protein levels of cell lysates. Eosinophils (1×10^7 cells in 1 ml) were stimulated with TNF (10 ng/ml), IL-5 (1 ng/ml), and TNF + IL-5 for the indicated periods of time, and lysed by several rounds of freezing and thawing. The eotaxin protein in cell lysates was assessed by a sandwich ELISA procedure. Controls indicate unstimulated eosinophils. Duplicate cultures were set up for each sample. Data represent the average of two independent experiments.



TNF or IL-5 augmented synthesis of eotaxin protein. Simultaneous treatment with TNF and IL-5 slightly decreased eotaxin protein expression, yet the combined effect was not as pronounced on the mRNA level, as seen in Figures 1e and 2.

Discussion

In this study we examined eotaxin gene expression in peripheral blood eosinophils in response to TNF and IL-5 primarily by *in situ* hybridization. Our data show that TNF induced eotaxin mRNA in a dose-dependent manner. This result is in agreement with other studies on eotaxin gene expression, in which several cell lines produce eotaxin mRNA in response to TNF (34, 35, 37). Recent cloning of a genomic eotaxin gene has demonstrated that its promoter contains nuclear factor (NF)- κ B binding element (49). This finding supports the fact that TNF may transactivate eotaxin gene through NF- κ B activation. Thus, TNF may be an important regulator to induce eotaxin gene expression and contribute eosinophil influx into the tissues. Our results also indicated that approximately 37% of eosinophils produced eotaxin mRNA in response to TNF (10 ng/ml). Although hypodense eosinophils are known to be more activated than normodense cells (40) and seem to occur in higher ratio in the patients' blood used for these experiments, we do not know which of two populations of eosinophils is more likely to express eotaxin gene.

Our data reveal that IL-5 alone was able to induce eotaxin mRNA. In accordance with our results, it has been observed that IL-3, which also acts as a survival factor for eosinophils, was able to induce eotaxin mRNA in eosino-

phils (42). In contrast, IL-5 inhibited TNF-induced eotaxin mRNA expression. A similar result was obtained from Southern blot hybridization following RT-PCR. We failed to obtain more quantitative data for eotaxin mRNA expression using a ribonuclease protection assay, mainly due to poor availability of RNA from the limited number of peripheral eosinophils, although a faint signal was obtained only from TNF-treated cells (data not shown). Unlike the induction of eotaxin mRNA by TNF, a considerable amount of eotaxin protein was constitutively produced in unstimulated eosinophils even if TNF or IL-5 caused enhanced eotaxin protein expression. Nakajima and colleagues have recently revealed that eotaxin protein is preformed and stored in specific granules of freshly isolated eosinophils, and that the preformed chemokine is secreted in response to appropriate stimuli (41). The existence of the preformed chemokine in unstimulated eosinophils would explain the high levels of eotaxin protein shown in our study. Moreover, the inhibitory effect on TNF-mediated eotaxin protein production by IL-5 did not appear to be as significant as that on eotaxin mRNA levels. Thus, the inhibitory effect could be relatively marginal on protein levels. Although eosinophils are capable of producing eotaxin either constitutively or in response to stimuli, as in this study and others (41, 42), the eotaxin protein levels appear to have less physiologic relevance. Indeed the primary cellular source of eotaxin protein in allergic disease states has been shown to be epithelial cells in extensive *in vivo* studies (5, 17, 50). The possible mechanisms by which IL-5 downregulates the expression of eotaxin mRNA in the presence of TNF is hardly envisionable at this time. IL-5 binding to its receptor on eosinophils is reported to

activate signaling molecules such as Jak/STAT family proteins (51) and protein kinases/protein phosphatases (38). On the other hand, TNF is known to exert its functions, including NF- κ B activation and apoptosis, through differential interaction of distinct signaling molecules such as TRAF1/2 and TRADD (52). Hence, there might exist cross-talks between signaling molecules in cascade events induced by the two cytokines.

The physiologic significance of IL-5 inhibition of TNF-induced eotaxin gene expression is not clear. We expected that IL-5 would give rise to an additive and/or synergistic effect on eotaxin mRNA expression in the presence of TNF. The synergism by the two cytokines would provide the local environment in which eosinophils are more abundant and live longer to sustain their functions in inflamed tissues. In our results, either TNF or IL-5 alone is able to induce eotaxin mRNA expression, but the latter reverses the former-mediated effect upon the simultaneous treatment. Several lines of evidence illustrate that IL-5 prolongs eosinophil survival *in vitro* (53, 54), activates normodense to hypodense eosinophils (40), and directly causes eosinophil degranulation (2), whereas TNF promotes eosinophil influx (18, 19, 22) and eosinophil death (our unpublished observation) and causes eosinophil degranulation (2). Thus, the two cytokines acting on eosinophils have not only functional redundancy but also opposite effects. One possibility is that IL-5 would counteract its positive effect on eosinophil survival by decreasing eotaxin gene expression in the inflamed state so as to maintain the balanced eosinophil numbers, as TNF does its positive effect on eosinophil recruitment by promoting eosinophil death. This fact reflects the complex physiology of eosinophils in response to cytokines. It would be interesting to examine whether eosinophil survival factors such as GM-CSF and IL-3 are also able to alter eotaxin gene expression in this context.

Acknowledgments: This work was supported by KOSEF 96-0403-07-01-3, Korea. The authors thank Ms. Myung Ran Lee for assistance with eosinophil isolation.

References

1. Frigas, E., and G. J. Gleich. 1986. The eosinophil and the pathophysiology of asthma. *J. Allergy Clin. Immunol.* 77:527-537.
2. Takafuji, S., T. Ohtoshi, H. Takizawa, K. Tadokoro, and K. Ito. 1996. Eosinophil degranulation in the presence of bronchial epithelial cells. *J. Immunol.* 156:3980-3985.
3. Rothenberg, M. E., J. A. MacLean, E. Pearlman, A. D. Luster, and P. Leder. 1997. Targeted disruption of the chemokine eotaxin partially reduces antigen-induced tissue eosinophilia. *J. Exp. Med.* 185:785-790.
4. Jose, P. J., D. A. Griffiths-Johnson, P. D. Collins, D. T. Walsh, R. Moqbel, N. F. Totty, O. Truong, J. J. Hsuan, and T. J. Williams. 1994. Eotaxin: a potent eosinophil chemoattractant. *J. Exp. Med.* 179:881-887.
5. Lamkhied, B., P. M. Renzi, S. Abi-Younes, E. A. Garcia-Zepeda, Z. Al-lakhverd, O. Ghaffar, M. D. Rothenberg, A. D. Luster, and Q. Hamid. 1997. Increased expression of eotaxin in bronchoalveolar lavage and airways of asthmatics contributes to the chemotaxis of eosinophils to the site of inflammation. *J. Immunol.* 159:4593-4601.
6. Okada, S., H. Kita, T. J. George, G. J. Gleich, and K. M. Leiferman. 1997. Transmigration of eosinophils through basement membrane components *in vitro*: synergistic effects of platelet-activating factor and eosinophil-active cytokines. *Am. J. Respir. Cell Mol. Biol.* 16:455-463.
7. Gonzalo, J. A., C. M. Lloyd, L. Kremer, E. Finger, C. Martinez-A. C., M. H. Siegelman, M. Cybulsky, and J. C. Gutierrez-Ramos. 1996. Eosinophil recruitment to the lung in a murine model of allergic inflammation: the role of T cells, chemokines, and adhesion receptors. *J. Clin. Invest.* 98:2332-2345.
8. Mould, A. W., K. I. Matthaei, I. G. Young, and P. S. Foster. 1997. Relationship between interleukin-5 and eotaxin in regulating blood and tissue eosinophilia in mice. *J. Clin. Invest.* 99:1064-1071.
9. Gao, J.-L., A. I. Sen, M. Kitaura, O. Yoshie, M. E. Rothenberg, P. M. Murphy, and A. D. Luster. 1996. Identification of a mouse eosinophil receptor for the CC chemokine eotaxin. *Biochem. Biophys. Res. Commun.* 223:679-684.
10. Daugherty, B. L., S. J. Siciliano, J. A. DeMartino, L. Malkowitz, A. Sirotna, and M. S. Springer. 1996. Cloning, expression, and characterization of the human eosinophil eotaxin receptor. *J. Exp. Med.* 183:2349-2354.
11. Heath, H., S. Qin, P. Rao, L. Wu, G. LaRosa, N. Kassam, P. D. Ponath, and C. R. Mackay. 1997. Chemokine receptor usage by human eosinophils: the importance of CCR3 demonstrated using an antagonistic monoclonal antibody. *J. Clin. Invest.* 99:178-184.
12. Ponath, P. D., S. Qin, T. W. Post, J. Wang, L. Wu, N. P. Gerard, W. Newman, C. Gerard, and C. R. Mackay. 1996. Molecular cloning and characterization of a human eotaxin receptor expressed selectively on eosinophils. *J. Exp. Med.* 183:2437-2448.
13. Yamada, H., K. Hirai, M. Miyamasu, M. Iikura, Y. Misaki, S. Shoji, T. Takashi, T. Kasahara, Y. Morita, and K. Ito. 1997. Eotaxin is a potent chemotactic for human basophils. *Biochem. Biophys. Res. Commun.* 231:365-368.
14. Sallusto, F., C. R. Mackay, and A. Lanzavecchia. 1997. Selective expression of the eotaxin receptor CCR3 by human T helper 2 cells. *Science* 277:2005-2007.
15. Kitaura, M., T. Nakajima, T. Imai, S. Harada, C. Combadiere, H. L. Tiffany, P. M. Murphy, and O. Yoshie. 1996. Molecular cloning of human eotaxin, an eosinophil-selective CC chemokine, and identification of a specific eosinophil eotaxin receptor, CC chemokine receptor 3. *J. Biol. Chem.* 271:7725-7730.
16. Ponath, P. D., S. Qin, D. J. Ringler, I. Clark-Lewis, J. Wang, N. Kassam, H. Smith, X. Shi, J. A. Gonzalo, W. Newman, J. C. Gutierrez-Ramos, and C. R. Mackay. 1996. Cloning of the human eosinophil chemoattractant, eotaxin: expression, receptor binding, and functional properties suggest a mechanism for the selective recruitment of eosinophils. *J. Clin. Invest.* 97:604-612.
17. Mattoli, S., M. A. Stacey, G. Sun, A. Bellini, and M. Marini. 1997. Eotaxin expression and eosinophilic inflammation in asthma. *Biochem. Biophys. Res. Commun.* 236:299-301.
18. Ming, W. J., L. Bersani, and A. Mantovani. 1987. Tumor necrosis factor is chemotactic for monocyte and polymorphonuclear leukocytes. *J. Immunol.* 138:1469-1474.
19. Pober, J. S., M. A. Gimbrone, Jr., L. A. Lapierre, D. L. Mendrick, W. Fiers, R. Rothlein, and T. A. Springer. 1986. Overlapping patterns of activation of human endothelial cells by interleukin-1, tumor necrosis factor and immune interferon. *J. Immunol.* 137:1893-1896.
20. Bevilacqua, M. P., J. S. Pober, D. L. Mendrick, R. S. Cotran, and M. A. Gimbrone, Jr. 1987. Identification of an inducible endothelial-leukocyte adhesion molecule. *Proc. Natl. Acad. Sci. USA* 84:9238-9242.
21. Osborn, L., C. Hession, R. Tizard, C. Vassallo, S. Lohowskyj, G. Chi-Rosso, and R. Lobb. 1989. Direct expression cloning of vascular cell adhesion molecule 1, a cytokine-induced endothelial protein that binds to lymphocytes. *Cell* 59:1203-1211.
22. Godding, V., J. M. Stark, J. B. Sedgwick, and W. W. Busse. 1995. Adhesion of activated eosinophils to respiratory epithelial cells is enhanced by tumor necrosis factor- α and interleukin-1 β . *Am. J. Respir. Cell Mol. Biol.* 13:555-562.
23. Broudy, V. C., K. Kaushansky, G. M. Segal, J. M. Harlan, and J. W. Adamson. 1986. Tumor necrosis factor type α stimulates human endothelial cells to produce granulocyte/macrophage colony-stimulating factor. *Proc. Natl. Acad. Sci. USA* 83:7467-7471.
24. Strieter, R. M., S. W. Chensue, M. A. Basha, T. J. Standiford, J. P. Lynch, M. Baggiolini, and S. L. Kunkel. 1990. Human alveolar macrophage gene expression of interleukin-8 by tumor necrosis factor- α , lipopolysaccharide and interleukin-1 β . *Am. J. Respir. Cell Mol. Biol.* 2:321-326.
25. Marini, M., M. Soloperto, M. Mezzetti, A. Fasoli, and S. Mattoli. 1991. Interleukin-1 binds to specific receptors on human bronchial epithelial cells and upregulates granulocyte/macrophage colony-stimulating factor synthesis and release. *Am. J. Respir. Cell Mol. Biol.* 4:519-524.
26. Wang, J. H., J. L. Devalia, C. Xia, R. J. Sapsford, and R. J. Davies. 1996. Expression of RANTES by human bronchial epithelial cells *in vitro* and *in vivo* and the effect of corticosteroids. *Am. J. Respir. Cell Mol. Biol.* 14:27-35.
27. Metcalf, D., C. G. Begley, G. R. Johnson, N. A. Nicola, M. A. Vadas, A. F. Lopez, D. J. Williamson, G. G. Wong, S. C. Clark, and E. A. Wang. 1986. Biologic properties *in vitro* of a recombinant human granulocyte/macrophage colony-stimulating factor. *Blood* 67:37-45.
28. Lopez, A. F., D. J. Williamson, J. R. Gamble, C. G. Begley, J. M. Harlan, S. J. Klebanoff, A. Waltersdorff, G. Wong, S. C. Clark, and M. A. Vadas. 1986. Recombinant human granulocyte/macrophage colony-stimulating factor stimulates *in vitro* mature human neutrophil and eosinophil function, surface receptor expression and survival. *J. Clin. Invest.* 78:1220-1228.
29. Silberstein, D. S., W. F. Owen, J. C. Gasson, J. F. DiPersio, D. W. Golde, J. C. Bina, R. Soberman, K. F. Austen, and J. R. David. 1986. Enhancement of human eosinophil cytotoxicity and leukotriene synthesis by biosynthetic (recombinant) granulocyte/macrophage colony-stimulating factor. *J. Immunol.* 137:3290-3294.

30. Watson, M. L., D. Smith, A. D. Bourne, R. C. Thompson, and J. Westwick. 1993. Cytokines contribute to airway dysfunction in antigen-challenged guinea pigs: inhibition of airway hyperreactivity, pulmonary eosinophil accumulation, and tumor necrosis factor generation by pretreatment with an interleukin-1 receptor antagonist. *Am. J. Respir. Cell Mol. Biol.* 8:365-369.
31. Moncada, S., R. M. Palmer, and E. A. Higgs. 1991. Nitric oxide: physiology, pathophysiology and pharmacology. *Pharmacol. Rev.* 43:109-142.
32. Fujisawa, T., R. Abu-Ghazaleh, H. Kita, C. J. Sanderson, and G. J. Gleich. 1990. Regulatory effect of cytokines on eosinophil degranulation. *J. Immunol.* 144:642-646.
33. Whitcomb, E. A., J. H. Wesolek, and S. H. Pincus. 1989. Modulation of human peripheral blood eosinophil function by tumor necrosis factor- α . *Int. Arch. Allergy Appl. Immunol.* 89:250-255.
34. Lilly, C. M., H. Nakamura, H. Kesselman, C. Nagler-Anderson, K. Asano, E. A. Garcia-Zepeda, M. E. Rothenberg, J. M. Drazen, and A. D. Luster. 1997. Expression of eotaxin by human lung epithelial cells: induction by cytokines and inhibition by glucocorticoids. *J. Clin. Invest.* 99:1767-1773.
35. Rothenberg, M. E., A. D. Luster, and P. Leder. 1995. Murine eotaxin: an eosinophil chemoattractant inducible in endothelial cells and in interleukin 4-induced tumor suppression. *Proc. Natl. Acad. Sci. USA* 92:8960-8964.
36. Nakamura, H., K. J. Haley, T. Nakamura, A. D. Luster, and C. M. Lilly. 1998. Differential regulation of eotaxin expression by TNF- α and PMA in human monocytic U937 cells. *Am. J. Physiol.* 275:601-610.
37. Bartels, J., C. Schluter, E. Richter, N. Noso, R. Kulke, E. Christophers, and J. M. Schroder. 1996. Human dermal fibroblasts express eotaxin: molecular cloning, mRNA expression, and identification of eotaxin sequence variants. *Biochem. Biophys. Res. Commun.* 225:1045-1051.
38. Pazdrak, K., T. Adachi, and R. Alam. 1997. Src homology 2 protein tyrosine phosphatase (SHPTP2)/Src homology 2 phosphatase 2 (SHP2) tyrosine phosphatase is a positive regulator of the interleukin-5 receptor signal transduction pathways leading to the prolongation of eosinophil survival. *J. Exp. Med.* 186:561-568.
39. Rothenberg, M. E., R. Ownbey, P. D. Mehlhob, P. M. Loiselle, M. van de Rijn, J. V. Bonventre, H. C. Oettgen, P. Leder, and A. D. Luster. 1996. Eotaxin triggers eosinophil-selective chemotaxis and calcium flux via a distinct receptor and induces pulmonary eosinophilia in the presence of interleukin-5 in mice. *Mol. Med.* 2:334-348.
40. Rothenberg, M. E., W. F. Owen, Jr., D. S. Silberstein, R. J. Soberman, A. L. Sheffer, K. F. Austen, and R. L. Stevens. 1988. Cytokine regulation of human eosinophil viability, density and function. *J. Allergy Clin. Immunol.* 81:209. (Abstr.)
41. Nakajima, T., H. Yamada, M. Ikura, M. Miyamasu, S. Izumi, H. Shida, K. Ohta, T. Imai, O. Yoshie, M. Mochizuki, J.-M. Schroder, Y. Morita, K. Yamamoto, and K. Hirai. 1998. Intracellular localization and release of eotaxin from normal eosinophils. *FEBS Lett.* 434:226-230.
42. Garcia-Zepeda, E. A., M. E. Rothenberg, R. T. Ownbey, J. Celestin, P. Leder, and A. D. Luster. 1996. Human eotaxin is a specific chemoattractant for eosinophil cells and provides a new mechanism to explain tissue eosinophilia. *Nat. Med.* 2:449-456.
43. Gartner, I. 1980. Separation of human eosinophils in density gradients of polyvinylpyrrolidone-coated silica gel (Percoll). *Immunology* 40:133-136.
44. Miltenyi, S., and S. Pfluger. 1992. High gradient magnetic cell sorting. In *Flow Cytometry and Cell Sorting*. A. Radbruch, editor. Springer-Verlag, Hong Kong. 141-152.
45. Yokoyama, W. M. 1991. Flow cytometry analysis using the Becton Dickinson FACScan. In *Current Protocols in Immunology*. J. E. Coligan, editor. Green Publishing Associates and Wiley-Interscience, New York. Unit 5.4.
46. Chomczynski, P., and N. Sacchi. 1987. Single step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* 162:156-159.
47. Chung, I. Y., J. Kwon, and E. N. Benveniste. 1992. Role of protein kinase C activity in tumor necrosis factor- α gene expression: involvement at the transcriptional level. *J. Immunol.* 149:3894-3902.
48. Scott Young, W. 1992. In situ hybridization with oligonucleotide probes. In *In Situ Hybridization*. D. G. Wilkinson, editor. Oxford University Press, Oxford, UK. Ch. 3.
49. Hein, H., C. Schluter, R. Kulke, E. Christophers, J. M. Schroder, and J. Bartels. 1997. Genomic organization, sequence, and transcriptional regulation of the human eotaxin gene. *Biochem. Biophys. Res. Commun.* 237:537-542.
50. Humbles, A. A., D. M. Conroy, S. Marleau, S. M. Rankin, R. T. Palframan, A. E. I. Proudfoot, T. N. C. Wells, D. Li, P. K. Jeffery, D. A. Griffiths-Johnson, T. J. Williams, and P. J. Jose. 1997. Kinetics of eotaxin generation and its relationship to eosinophil accumulation in allergic airways disease: analysis in guinea pig model *in vivo*. *J. Exp. Med.* 186:601-612.
51. Bruggen, T., E. Caldenhoven, D. Kanter, P. Coffey, J. A. M. Raaijmakers, J. J. Lammers, and L. Koenderman. 1995. Interleukin-5 signaling in human eosinophils involves JAK2 tyrosine kinase and Stat1 α . *Blood* 85:1442-1448.
52. Hsu, H., H.-B. Shu, M.-G. Pan, and D. V. Goeddel. 1996. TRADD-TRAF2 and TRADD-FADD interactions define two distinct TNF receptor 1 signal transduction pathways. *Cell* 84:299-308.
53. Yamaguchi, Y., T. Suda, S. Ohta, K. Tominaga, Y. Miura, and T. Kasahara. 1991. Analysis of the survival of mature human eosinophils: interleukin-5 prevents apoptosis in mature human eosinophils. *Blood* 78:2542-2547.
54. Stern, M., L. Meagher, J. Savill, and C. Haslett. 1992. Apoptosis in human eosinophils: programmed cell death in the eosinophil leads to phagocytosis by macrophages and is modulated by IL-5. *J. Immunol.* 148:3543-3549.